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HTLV-III *env* Gene Products Synthesized in E. coli Are Recognized by Antibodies Present in the Sera of AIDS Patients

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Summary

The envelope gene of HTLV-III, the retrovirus directly linked to AIDS, encodes a protein of 856 amino acids. Our sequence analysis of the cloned HTLV-III (HXB-3) *env* gene and its comparison with other isolates reveal significant divergence, especially in the external portion of this protein. A large segment of the *env* gene (1800 bp) was inserted into the expression vector pEV-vrf3, and a corresponding 68 kd protein, which encompasses both the extracellular and the membrane-associated regions of the native protein, was produced in E. coli. Several smaller polypeptides, which appear to be internal initiation products, were also produced. All 50 AIDS patient sera obtained from different locations in the United States specifically recognized the bacterially synthesized envelope proteins, as judged by Western blots. This suggests that these proteins will be useful for the diagnosis of HTLV-III infection and possibly as a vaccine against AIDS.

Introduction

A retrovirus termed HTLV-III, LAV, or ARV-2 has been isolated from cultured T cells of several patients with acquired immune deficiency syndrome (AIDS) or from white blood cells of persons at risk for the disease (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984; Montagnier et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984). The virus can be propagated in an immortalized human neoplastic cell line (HT) and in several clones of this cell line (Popovic et al., 1984). Seroepidemiological assays using this virus showed a close correlation between AIDS and antibodies to HTLV-III antigens (Gallo et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984). In addition, nearly 85% of patients with lymphadenopathy syndrome and a significant proportion of asymptomatic homosexual men in AIDS endemic areas also carry circulating antibodies to HTLV-III. All these data implicate HTLV-III as the etiological agent for AIDS. The need for sensitive and rapid methods for the diagnosis of AIDS and for its prevention by vaccination to relieve the current global spread of this disease is clear. Toward this end, we have expressed a large segment of the HTLV-III *env* gene in E. coli, and in this report, we demonstrate the utility of the *env* proteins made in bacteria for detecting antibodies to HTLV-III in sera obtained from AIDS patients.

Results

Molecular Cloning and Nucleotide Sequence Analysis of the HTLV-III Proviral Genome

The integrated proviral genome of HTLV-III was recently cloned from the genomic DNA of H9 cells infected with HTLV-III (Shaw et al., 1984). Since the HTLV-III provirus was found to lack Xba I restriction sites, a genomic library was constructed using Xba I digested H9/HTLV-III DNA. Screening of this library with HTLV-III cDNA probes yielded several clones, one of which, termed HXB-3 was used in the present study. The complete nucleotide sequence of the proviral genome has been determined in our lab (Reddy, unpublished data). The sequence of other isolates has recently been reported from other laboratories (Ratner et al., 1985; Sanchez-Pescadov et al., 1985; Wain-Hobson et al., 1985). The nucleotide sequence of the envelope gene used in the present study is shown in Figure 1. The open reading frame encoding the *env* gene is 863 amino acids long and contains an ATG codon at the eighth position from the 5′ end of the reading frame. If this ATG were used as the initiator codon, the opening reading frame would code for a 97,200 dalton protein. The sequence agrees well with the recently published sequences of two other HTLV-III, LAV, and ARV-2 sequences. The predicted product of the *env* gene shares many features in common with the envelope gene products of other retroviruses. The amino terminus contains a short stretch of hydrophobic amino acids (18–29) that constitutes a potential signal sequence. Also common with other retroviral envelope proteins is the presence of putative processing sites (504–511) in the middle of the protein that separate the extracellular polypeptide from the membrane-bound portion. The latter includes a hydrophobic transmembrane segment (667–705) followed by a hydrophilic anchor sequence (725–746). The HTLV-III envelope precursor differs from the other retroviral *env* precursors in that it contains an additional stretch of 180 amino acids at the carboxyl terminus.

Polymorphism within the Envelope Regions of AIDS-Associated Viruses

The recent publication of the nucleotide sequences of HTLV-III, LAV, and ARV-2 (Ratner et al., 1985; Sanchez-Pescadov et al., 1985; Wain-Hobson et al., 1985) allows a detailed comparison of these various isolates obtained from AIDS patients from different parts of the world. HTLV-III clones were isolated from lymphocytes of an AIDS patient on the east coast of the United States, ARV-2 was isolated from a patient in California, and LAV was isolated from a patient in France. A comparison of the sequence

[illegible]

Figure 1. Nucleotide Sequence of the Envelope Gene of the HTLV-III Proviral Genome (HXB-3)

The sequence proceeding in the 5' to 3' direction has the same polarity as the genomic RNA. The amino acid sequence deduced from the open reading frame is given below the nucleotide sequence. Pertinent restriction sites present within this sequence are indicated.

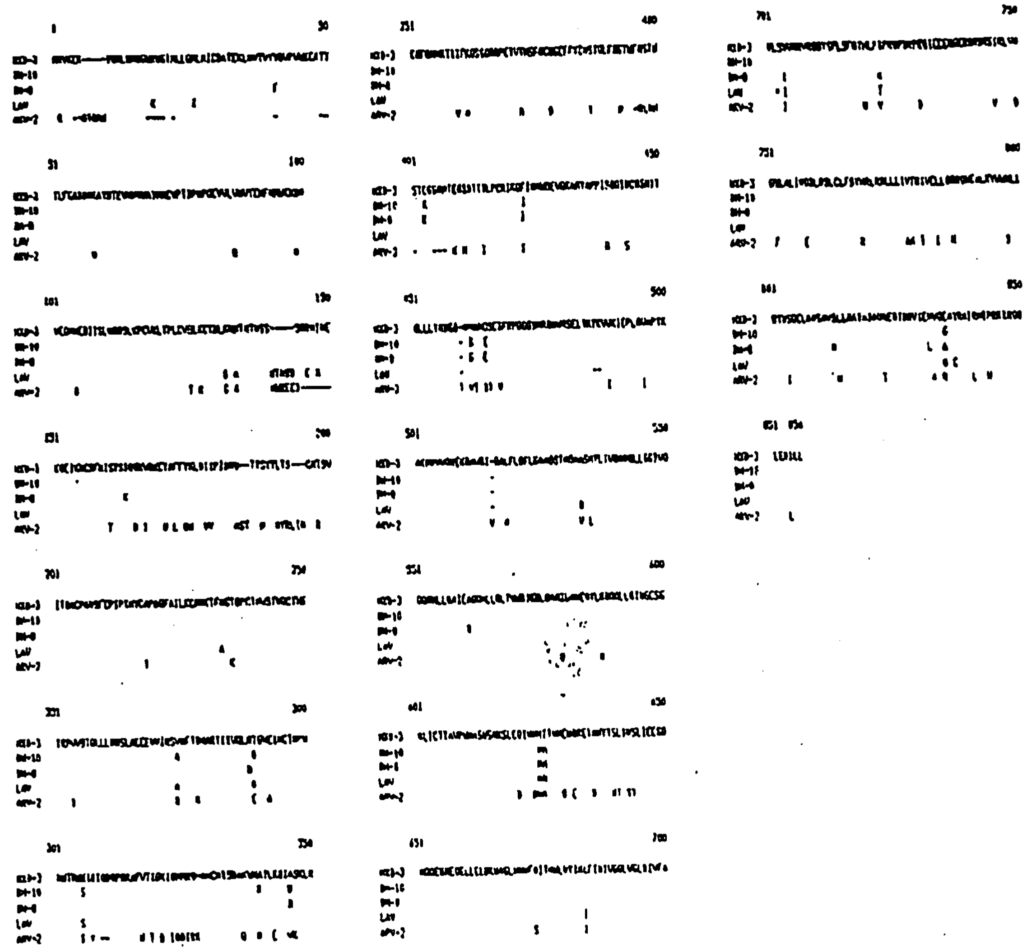

Figure 2. Sequence Comparison of AIDS Retroviral Isolates

Amino acid sequences of the five retroviral isolates are aligned to give maximum homology. (–) indicates deletion of one amino acid. An empty space denotes identity with HXB-3 sequence. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp, Y, Tyr.

data confirms the earlier observations made using restriction enzyme site analysis, which showed approximately 10% variation (Shaw et al., 1984). The present analysis indicates that the various isolates show the greatest conservation in the *gag* and *pol* regions (data not shown), while they most diverge in the *env* region. A comparison of the four *env* protein sequences, as deduced from their nucleotide sequences, is presented in Figure 2. As shown in Figure 2, the ARV-2 protein differs most drastically from the other three sequences. Within the *env* region, the highest level of divergence is in the extracellular portion of the protein.

Construction of Bacterial Expression Plasmids for the Envelope Gene

Segments of the HTLV-III *env* gene were expressed in E. coli by inserting restriction fragments isolated from the cloned proviral genome into the versatile pEV-vrf (variable reading frame) expression plasmids (Crowl et al., 1985). These plasmids are derivatives of pBR322 that contain the phage lambda P_L promoter, a synthetically-derived ribosome-binding site, and convenient cloning sites (Eco RI, Bam HI, Cla I, and Hind III) just downstream of the initiation codon. A set of three pEV-vrf plasmids was constructed to accommodate all three translational reading frames. The P_L promoter is regulated by a temperature-sensitive cI repressor encoded on the compatible plasmid pRK248cIts (Bernard and Helinski, 1979). These expression plasmids have been used to produce substantial amounts of several heterologous proteins in E. coli, including v-*bas* p21 (Lacal et al., 1984) and murine interleukin-1 (Lomedico et al., 1984).

The coding sequences for amino acid residues 44–640 of the *env* protein are located between the Kpn I and the Hind III sites on the restriction map shown in Figure 3. Aside from the location of these convenient restriction sites, these sequences were chosen for bacterial expression experiments because they did not include the amino-terminal signal peptide or the hydrophobic transmembrane segment at the carboxyl end. These sequences

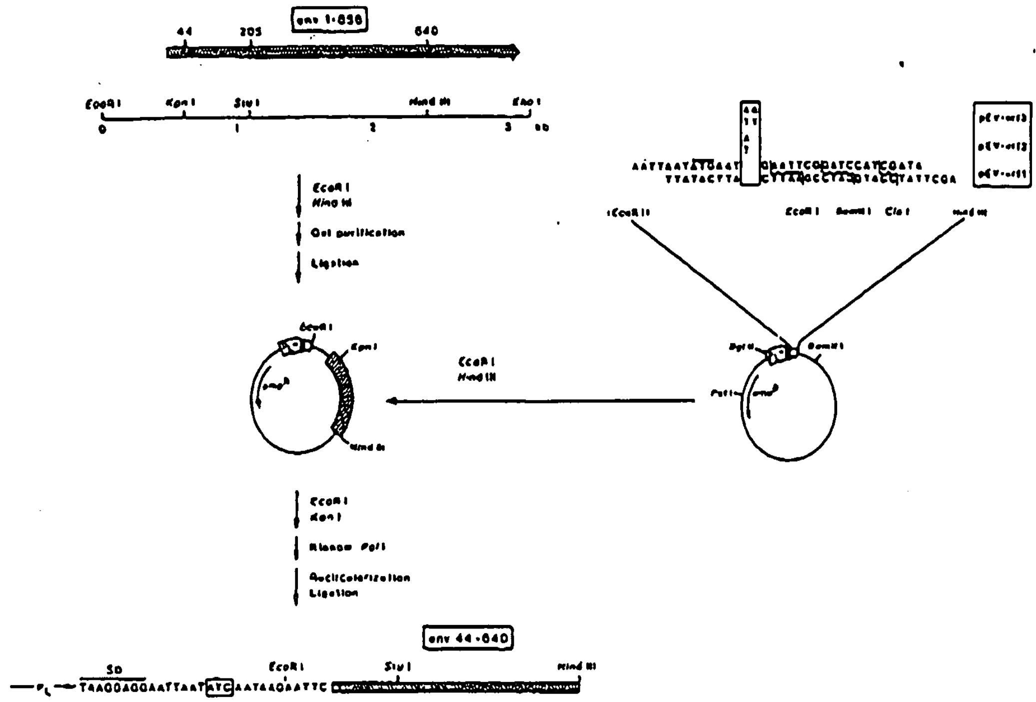

Figure 3. Construction of the PEV/env44–640 Expression Plasmids
(Upper left) a simplified restriction site map of the 3.15 kb Eco RI-Xho I segment of the HTLV-III genome that contains the *env* coding region (cross-hatched arrow). (Right) the structure and pertinent sequences of the pEV-vrf plasmids. The solid black region represents the synthetic ribosome binding site sequences upstream of the ATG initiation codon (overlined). See Experimental Procedures for a detailed description of the *env* expression plasmid constructions.

were excluded to avoid possible toxicity problems that can occur when hydrophobic proteins are overproduced in bacterial cells. To construct an expression plasmid that would direct the synthesis of this segment of the *env* gene product (designated pEV/env 44–640), an intermediate construction was first made by inserting a 2400 bp Eco RI-Hind III fragment between the Eco RI and Hind III sites in the pEV-vrf plasmids. The HTLV-III sequences (600 bp) between the Eco RI and the Kpn I site were then removed from the intermediate construction as shown in Figure 3. These plasmid constructions were carried out with all three pEV-vrf plasmids so that subsequent deletions could be made and the correct reading frame could be maintained. In addition, the constructions made in the incorrect reading frames served as important controls in the expression experiments described below. A second set of expression plasmids was made in a similar fashion by deleting sequences between Eco RI and Stu I sites, which occur 483 bp downstream to the Kpn I site in the *env* gene. Again, these deletions (designated pEV/env 205–640) were made in all three reading frames. The translation termination codon used in all of the *env* expression plasmids is presumably an inframe TAA located 23 bp downstream of the Hind III site in the plasmid. Thus, eight amino acid residues at the carboxyl terminus are encoded by pBR322 sequences.

Cultures of E. coli containing the *env* expression plasmids were grown and induced as described in Experimental Procedures. The bacterial proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the HTLV-III *env* proteins were detected by Western blot analysis. The protein blots were treated with antisera to HTLV-III proteins isolated either from immunized rabbits or from AIDS patients previously shown to contain high titer antibodies to HTLV-III antigens. This was followed by incubation with ^{125}I-labeled Staphylococcus aureus protein A, washing, and autoradiography. Similar results were obtained with both sera except that human serum was found to contain much higher titers of anti-HTLV-III antibodies and was devoid of all background reactivity with the E. coli proteins. For this reason, human antibodies were used in all subsequent experiments.

Figure 4 shows the pattern of reactivity of the *env* proteins synthesized in bacteria with anti-HTLV-III antibodies. The open reading frame in pEV3/env 44–640 encodes a protein that should migrate as a 68 kd band on the gel. In fact, a 68 kd band is observed in the lane corresponding to the induced cells containing pEV3/env 44–640 (Figure 4, lane C). However, in addition to the 68 kd band, proteins of 35 kd, 25 kd, and 17 kd, which specifically cross-reacted with anti-HTLV-III antibodies, are synthesized. No HTLV-III cross-reacting bands are evident in the uninduced control (Figure 4, lane b) or in a second negative control sample (Figure 4, lane a) of induced cells containing a plasmid that directs the synthesis of the v-*bas* p21 oncogene product (Lacal et al., 1984). The appearance of multiple bands

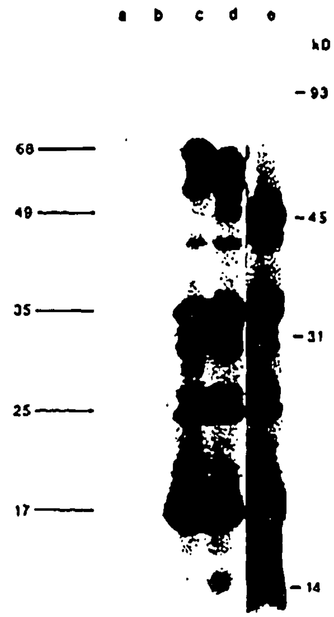

Figure 4. Western Blot Analysis of *env* Coded Antigens Produced in E. coli

Total bacterial proteins were resolved by SDS-PAGE, were electroblotted onto a nitrocellulose filter, and *env*-encoded proteins were detected by reacting with human sera as described in Experimental Procedures. (a) negative control, cells containing pJCL-E30 (p21T) (Lacal et al., 1984) induced at 42°C for 2 hr; (b) uninduced control, cells containing pEV3/env44–640 maintained at 30°C; (c) pEV3/env44–640; (d) pEV1/env44–640; and (e) pEV3/env205–640 induced at 42°C for 2 hr.

synthesized from the *env* gene sequences was an unexpected result. Another unexpected result was the synthesis of *env* gene products from the plasmid (pEV1/env 44–640) where the insert was placed in the wrong reading frame with respect to the initiation codon immediately downstream of the P_L promoter (Figure 4, lane d). In this case, a 63 kd protein is synthesized in addition to the 35 kd, 25 kd, and 17 kd proteins. These results could be readily explained by examining the nucleotide sequence of the envelope gene (Figure 1). About 155 bases downstream of the Kpn I site is an ATG codon, initiation at which could account for the 63 kd protein with *env* determinants produced by the expression plasmid pEV1/env 44–640. Internal translation initiation is also the likely explanation for the appearance of the 35 kd, 25 kd, and 17 kd proteins. ATG codons that are preceded by Shine-Dalgarno sequences are found within the *env* coding region, and translation initiating at these sites would result in the synthesis of polypeptides of molecular weights consistent with those observed.

To test the above interpretation further, we constructed another deletion mutant in which the sequences between Kpn I and Stu I sites were removed. This expression plasmid, which encodes a protein of 49 kd, contains the coding sequences from amino acid positions 205–640. Analysis of the proteins induced in E. coli harboring this plasmid verified that these cells synthesize a 49 kd protein in addition to the 35 kd, 25 kd, and 17 kd proteins (Figure 4, lane e). We have also constructed an expression plasmid in which the P_L promoter, without the synthetic ribosome binding site, was placed upstream of the 520 bp Bgl II-Hind III fragment containing the *env* codons 467–640; thus, the first inframe methionine codon downstream of the promoter corresponds to codon 475. This plasmid directs the synthesis of a protein that comigrates with the 17 kd band on a Western blot (data not shown). These results indicate that sequences within the *env* gene can function as efficient translation initiation sites in E. coli. It is possible that these sites become accessible as reinitiation sites following premature termination because of ribosomal frameshifting. Such a mechanism has been well characterized for the case of the MS2 coat and lysis genes (Kastelein et al., 1982).

Screening of AIDS Patient Sera

Because anti-HTLV-III antibodies are found in more than 90% of the AIDS patients, it was of interest to see if the bacterially synthesized *env* gene products could be used as diagnostic tools for the detection of these antibodies. For this, total cell protein from an induced bacterial culture was fractionated by SDS-PAGE and was transferred to a nitrocellulose filter. Strips of the filter containing transferred proteins were reacted with 1000× diluted human sera, and the antigen–antibody complexes formed were detected by incubation of the strips with ^{125}I-labeled Staphylococcus aureus protein A followed by autoradiography. Prominent bands corresponding to reaction of the antibody with the 68 kd, 35 kd, 25 kd, and 17 kd proteins were consistently observed when the serum used was from patients with AIDS. The results of one such assay with 18 human sera obtained from the east coast of the United States are presented in Figure 5A. No reaction was observed with sera from healthy individuals (Figure 5A, lanes 4, 5, 9, 11, and 15; Figure 5B, lane 1) or from HTLV-I-infected individuals (Figure 5A, lane 1). AIDS patient sera, tested so far, derived from all parts of the United States, including California (Figure 5B), were found to be positive.

Discussion

We have determined the nucleotide sequence of the HTLV-III *env* gene and expressed a large segment of its product in E. coli as an approach to obtain specific proteins that could be used for the diagnosis of and vaccination against AIDS. The recombinant protein contains 611 amino acids and encompasses both the extracellular and the membrane-associated regions of the molecule. In addition to the 68 kd protein product, the bacteria also synthesize several smaller polypeptides that appear to be products of the envelope gene initiated at internal methionine codons. The bacterially synthesized proteins differ from the naturally occurring protein in several important

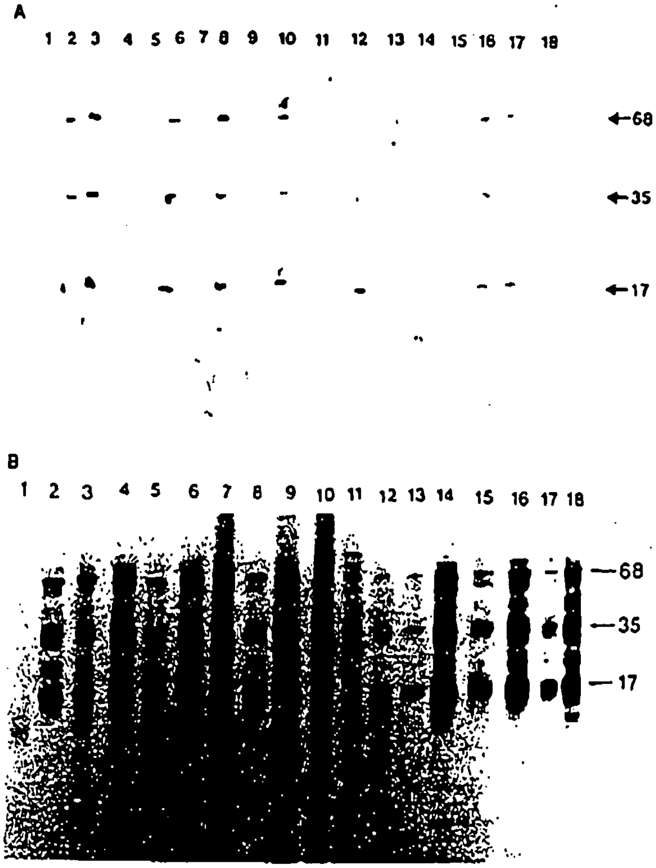

Figure 5. Recognition of Bacterially Synthesized HTLV-III Envelope Gene Products by Antibodies in AIDS Patient Sera

Bacterial lysates containing recombinant envelope proteins were subjected to Western blot analysis as described in Figure 4. Individual strips were then incubated with a 1000× dilution of individual sera followed by treatment with ^{125}I-labeled protein A as described in Experimental Procedures. (A) Serum samples were taken from the following donors: (lane 1) donor found to be HTLV-I(+) by ELISA using disrupted virus; (lanes 4, 5, 11, and 15) healthy, normal donors; (lanes 2, 3, 6, 8, 10, 12, 13, 14, 16, 17, and 18) AIDS patient sera from the east coast of USA. (B) Serum samples were from the following donors: (lane 1) normal, healthy donor; (lanes 2–18) AIDS patient sera collected from California.

respects. The bacterial products are smaller in size and are not modified after translation. The naturally occurring *env* gene product, on the other hand, is extensively glycosylated and is cleaved into two polypeptides. Despite these differences, the data presented here demonstrate that these recombinant proteins are efficiently recognized by human antibodies to HTLV-III. Two sets of AIDS patient sera, one obtained from the east coast of the United States and the other from California, were used to test their reactivity against the recombinant proteins using Western blot analysis. Both sets of patient sera reacted with the polypeptides produced in bacteria to approximately the same level, while normal sera or HTLV-I infected patient sera showed no reactivity. These results strongly suggest that these recombinant *env* proteins can serve as excellent diagnostic reagents for the detection of HTLV-III infection. The results also suggest that these antibodies are directed against the protein backbone of the molecule rather than against the carbohydrate moieties.

There are two important advantages of using recombinant *env* proteins, versus the viral protein, as a diagnostic reagent. The first is the economic feasibility of producing large amounts of the protein in bacteria. Second, unlike the viral preparations of the *env* protein, the recombinant proteins would be devoid of any human antigens that may reduce the reliability of such a diagnostic test.

Nucleotide sequence analysis of the envelope gene of HTLV-III has indicated that it encodes a protein with a predicted molecular weight of 97 kd. Comparison of the four sequences published recently (including those of LAV and ARV-2) demonstrates that all of these viruses are variants of the same virus. Sequence analysis also confirms earlier findings from restriction endonuclease mapping (Shaw et al., 1984) of the HTLV-III genomes, which showed a continuous spectrum ranging from closely identical to about 10% divergent among various isolates. The major coding regions of the *env* gene appears to have undergone greater divergence than those of the *gag* or *pol* genes. With respect to the envelope gene, HTLV-III and LAV are more closely related to each other than to the ARV-2 clone. Approximately 3% divergence was observed between the HTLV-III (HXB-3) and LAV amino acid sequences. However, among the three HTLV-III sequences (HXB-3, HB-10, and BH-8), the divergence was 1.6%. Approximately 17% divergence was observed between HXB-3 and ARV-2 sequences, which was most pronounced in the extracellular region of the envelope protein. The observed differences in sequence probably reflect divergent evolution of strains separated in time and geography. ARV-2 was recently isolated from the west coast of the United States. The HTLV-III isolates for which nucleotide sequences have been determined were all obtained from

the east coast about one year earlier. LAV was obtained from a patient in France at about the same time. Regardless of the reasons, the fact that such divergence can occur, and that it is more pronounced in the extracellular portion of the envelope protein, is an important consideration in the design of both diagnostic reagents and possible vaccines.

Of the AIDS patient sera (50 individual samples, 25 derived from the east coast and 25 derived from California) tested so far, 100% showed high reactivity with the recombinant proteins. These data suggest that there may be conserved epitopes within the *env* protein of various isolates against which the immune system can mount an antibody reaction. Sequence analysis of additional variants of HTLV-III, such as those isolated from Haitian patients (R. C. Gallo, personal communication), should help to define these conserved epitopes which could, in turn, lead to a better understanding of the evolution of this virus. An extensive survey using our recombinant proteins and several hundred patient sera derived from all over the world is currently in progress. It should yield more valuable information concerning the epidemiology of this lethal disease. If the notion that the human immune system can mount a response against conserved epitopes of the envelope molecule is correct, the observed divergence between the various isolates of HTLV-III may not pose a serious problem for the use of the recombinant protein as a vaccine. The 68 kd protein described here may be ideally suited for such a purpose since it encompasses a large portion of the gene product and has the important feature of containing both the extracellular hydrophilic region and the membrane-associated hydrophobic region. We surmise that the 17 kd protein observed in our Western blots contains residues 475–640 (initiating at the methionine codon at 475), which would include the membrane-associated segment of the *env* protein. It is interesting to note that this region is much less divergent among the variants compared in Figure 2. In fact, of the few differences between HXB-3 and ARV-2 within this region, most of the changes are conservative ones. Thus, we believe that this region contains a highly conserved epitope that may provide the best possibility as a wide-range diagnostic reagent.

Experimental Procedures

Molecular Cloning and Nucleotide Sequence Analysis

The integrated proviral genome of HTLV-III was recently cloned from the genomic DNA of H9 cells infected with HTLV-III (Shaw et al., 1984). The proviral genome that was obtained by using Xba I-digested H9/HTLV-III DNA contained two internal Eco RI sites within the viral genome and two additional sites in the cloning vector λJ1. These sites were used for further subcloning the three DNA fragments of 5.5 kb, 4.5 kb, and 1.1 kb into pBR322. Nucleotide sequence analysis of the proviral genome was determined by the chemical degradation method of Maxam and Gilbert (1980). For the sequence analysis, DNA inserts from the three subclones were isolated by electroelution and were further cleaved with appropriate restriction enzymes. The DNA fragments were labeled at their 5′ ends, with γ-^{32}P-ATP using polynucleotide kinase or at their 3′ ends with α-^{32}P-NTP by filling in with the Klenow fragment of DNA polymerase I. The DNA fragments labeled at the two ends were cleaved with a second enzyme, and the fragments labeled at a single end were purified on 5% acrylamide gels and were used for sequence analysis. For the sequence analysis of the *env* gene, a shotgun approach was used where the 4.5 Eco RI fragment was cleaved with one of the following enzymes: Bgl II, Hind III, Xho I, Ava II, Hinf I, and Sau 3A. The restriction fragments were labeled and sequenced as described above.

Expression Plasmids

Expression plasmids pEV-vrf 1, 2, and 3 have been described (Crowl et al., 1985). For the expression of the HTLV-III envelope gene, 1 μg of a 2400 bp Eco RI-Hind III DNA fragment, which was isolated from the cloned HTLV-III proviral genome by preparative agarose gel electrophoresis, was mixed with 0.1 μg of Eco RI-Hind III-digested vector DNA (pEV-vrf 1, 2, or 3). After heating at 65°C for 3 min, the mixtures were chilled on ice, and 20 μl ligation reactions were assembled, containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM DTT, 0.3 mM ATP, and 200 units of T4 DNA ligase. After incubation at 15°C for 4 hr, the reactions were terminated by heating at 65°C for 5 min. The ligation products were used to transform E. coli strain MC1061 (pRK248cIts). Transformants were selected on LB agar containing 50 μg/ml ampicillin at 30°C for 18 hr. Isolates containing the expected plasmid constructions were identified by restriction analysis. These intermediate constructions were then used to make pEV1, 2, and 3/env 44–640 by deleting the 600 bp between the Eco RI and Kpn I sites as described below.

Approximately 0.5 μg plasmid DNA was digested with Kpn I and Eco RI. The resulting termini were then treated with the Klenow fragment of DNA polymerase I in the presence of all four deoxyribonucleotides (at 100 μM) at 37°C for 30 min. This step results in "filling-in" of the 5′ overhang of the Eco RI terminus and in removing the 3′ overhang of the Kpn I terminus. Upon recircularization of the linear plasmid and blunt-end ligation of these termini, an Eco RI site is regenerated. Transformants containing plasmids with the expected deletion were identified by restriction analysis.

A second set of deletion derivatives, designated pEV/env 205–640 was constructed in a similar fashion. A portion of the linear plasmid that had been digested with Eco RI and Kpn I and treated with Klenow (see above) was further digested with Stu I. Again, upon recircularization and blunt-end ligation the Eco RI site was regenerated; however, an additional 483 bp of *env* coding sequences were removed.

Bacterial Growth and Induction of *env* Gene Expression

Cultures of E. coli MC1061 (Casadaban and Cohen, 1980) containing pRK248cIts and the *env* expression plasmids were grown in M9 medium containing 0.5% glucose and 0.5% casamino acids at 30°C to midlog phase. The cultures were divided and were either maintained at 30°C or transferred to 42°C to derepress the P_L promoter. After 2 to 3 hr at 42°C, the cultures were chilled on ice, and the cells were collected by centrifugation and processed as described below.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Cell pellets (approximately 10^8 cells) were resuspended in TG buffer (10 mM Tris (pH 7.4) and 10% glycerol), mixed with an equal volume of 2× sample buffer (Laemmli, 1970), incubated at 95°C for 5 min, and subjected to SDS-PAGE (Laemmli, 1970). For Western blot analysis, the proteins from the acrylamide gel were electroblotted onto a 0.1 μm nitrocellulose membrane (Schleicher and Schuell) for 16 hr at 50 V, in 12.5 mM Tris, 96 mM glycine, 20% methanol, 0.01% SDS at pH 7.5. Processing of the blot was carried out using the methods described by Towbin et al. (1979). For treatment with the human sera, the blots were incubated with a 1000× dilution of the sera in antibody buffer (20 mM sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl, 1% BSA, and 0.05% Tween 20) for 2–6 hr. The blots were then washed twice with phosphate-buffered saline containing 0.05% Tween 20 and were incubated with ^{125}I-labeled Staphylococcus aureus protein A for an additional period of 1 hr. The blot was then washed twice in PBS-Tween 20 buffer, dried, and autoradiographed.

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